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Introduction

1. **Microorganisms and the world**

   "If you don't like bacteria, you are on the wrong planet" says Dr. Stewart Brand. We are surrounded by different forms of micro-organisms such as; bacteria, fungi, viruses etc. The presence of microorganism is like a yin yang. The Yang associated has allowed the life on earth to flourish. The Yin correlates to the harmful effects of the microbes such as causing diseases. Infectious diseases are the second most cause of deaths worldwide (Sumi et al., 2014). In order to treat such diseases antibiotics are being used. The entire field of medicine was revolutionized by discovery of penicillin by Sir Alexander Fleming in 1929. Thereafter, the era of antibiotic started. Due to uncontrolled use of antibiotics and adaptation of microorganisms to the antibiotics the problem of antimicrobial resistance (AMR) has become grave. Micro-organisms have developed resistance to many natural as well as synthetic antibiotics. Antimicrobial resistance is one of the most serious global public health threats in this century (Prestinaci et al., 2015). It is estimated that AMR claims 7,00,000 lives per year and the number has been thought to increase to 10 million by the year 2050 (By & Neill, 2016).

2. **Tackling Antimicrobial Resistance (AMR) –**

   Tackling AMR is the need of the hour. The care has to be taken on different fronts such as – creating public awareness, lowering the numbers and amounts of the antibiotics used in disease treatment, agriculture and other environments, Sanitation and hygiene, and developing alternative drugs (By & Neill, 2016). Different strategies are being used to find alternatives for drugs currently used. These strategies include - Phage Therapy, Use of lysins, Antibodies, Probiotics, Immune stimulation and Antimicrobial proteins or peptides (By & Neill, 2016).

3. **Antimicrobial Peptides (AMPs)–**

From the above strategies mentioned AMPs have started gaining more attention. AMPs are natural antibiotics (Maróti Gergely et al., 2011). AMPs have a broad action spectrum of targeted organisms ranging from viruses to parasites (Park et al., 2017). AMPs often act as first line of defence against the invading pathogens (Salas et al., 2015). First AMP Gramicidin was discovered in the year 1939 (McKenna & Fiad, 2008). AMPs are of single gene products and small in size, they can be synthesized swiftly by the host using minimal input of energy and biomass (Broekaert et al., 1995). More than 5000 AMPs have been discovered till date (Park et al., 2017). Increasingly, AMPs are being consolidated as a new class of antibiotics, due to their broad mechanism of action, including their effectiveness against multi-resistant microorganisms (Meneguetti et al., 2017).

AMPs are classified majorly on the basis of origin and charges. Based on origin AMPs are grouped as plant, animal, bacterial, viral, insect, seed etc. origin peptides. Based on the net charge AMPs are classified as cationic, anionic and amphipathic peptides.

4. **Various sources of AMPs –**

AMPs are found in living organisms from all the domains of life. Following table enlists different AMPs from such wide array of sources.

No.	AMPs	Source	Reference
1	Cyclotides: Kalata B1 and B2	Plant	(Salas et al., 2015)
2	Shepherins		
3	Arietin		
4	Astacidin	Animals	(Li et al., 2012)
5	Bacteridines		
6	Nisin	Microbial	(Tiwari et al., 2009)
7	Staphylococcocin C55		(Garneau et al., 2002)
8	Penetratin	Synthetic	(Li et al., 2012)

Table 1 Examples of AMPs from Different Sources

5. **Mechanisms of Action of Peptides –**

In order to act on a bacterial cell, the peptides should first get attached to the cell. The attachment occurs due to the interaction of charges present on the cell and peptides. Once attached these molecules try to disrupt the membrane structures thus making the cell leaky and killing it. Common mechanisms by which the molecules act are as follows.

Sr. No.	Mechanism	Impact on bacterial cells
1	Carpet like	Coats a small area on the lipid bilayer which leads to formation of pores.
2	Aggregate formation	Effective molecules insert themselves vertically and form small spheres which leading to pore formations.
3	Toroidal Pore	Hydrophobic regions of the peptides get associated with centre part of the lipid bilayer.
4	Membrane Thinning	Peptides form gaps between lipid molecules and pull neighbouring molecules creating thin membranes

Table 2 Mechanism of AMP Action

(Bahar and Ren, 2013), (Brogden, 2005)

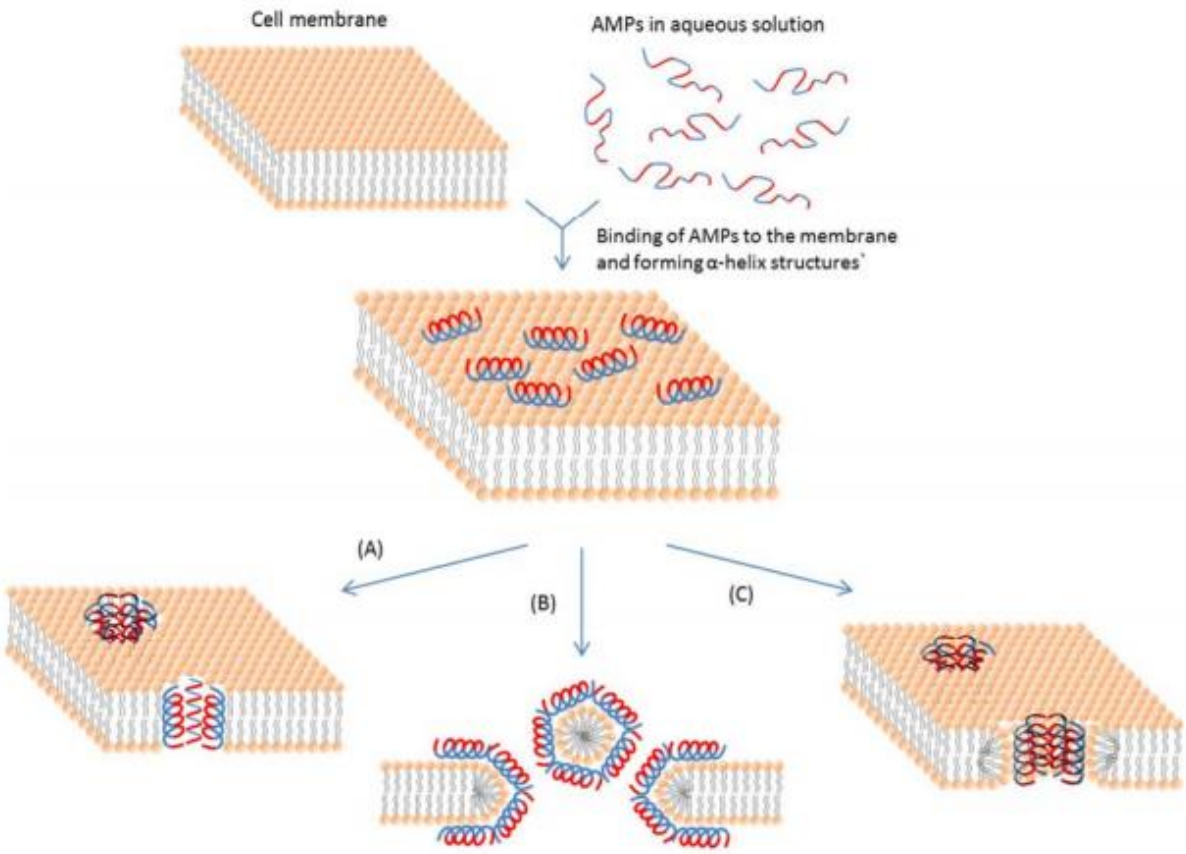

Figure 1 Schematic representation of Barrel Stave, Carpet and Torridal Pore action mechanisms of membrane-active AMPs – A. Barrel Stave model – AMPs insert themselves perpendicular B. Carpet Model – Small areas on the membrane get coated with AMPs (hydrophobic regions facing towards membrane), C. Toroidal pore model- This model resembles the Barrel-stave model, but AMPs are always in contact with phospholipid head groups of the membrane. The blue colour represents the hydrophobic portions of AMPs, while the red colour represents the hydrophilic parts of the AMPs.

(Bahar and Ren, 2013)

6. **Intracellular interactions of AMPs –** Some AMPs penetrate the cell membranes and gain entry in the cytoplasm. The cytoplasmic presence of AMPs allows them to interact with different targets within the cells. The targets include proteins, DNA, RNA, Ribosomes etc. This creates impairment of cellular functions.

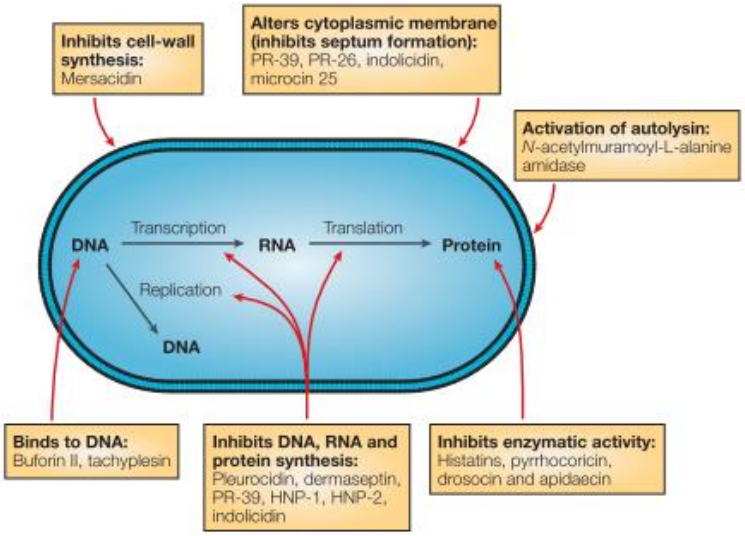
Inhibits cell-wall synthesis: Mersacidin
Alters cytoplasmic membrane (inhibits septum formation): PR-39, PR-26, indolicidin, microcin 25
Activation of autolysin: N-acetylmuramoyl-L-alanine amidase
DNA
Transcription
RNA
Translation
Protein
Replication
DNA
Binds to DNA: Buforin II, tachyplesin
Inhibits DNA, RNA and protein synthesis: Pleurocidin, dermaseptin, PR-39, HNP-1, HNP-2, indolicidin
Inhibits enzymatic activity: Histatins, pyrrhocoricin, drosocin and apidaecin

Figure 2 Intracellular Targets of AMPs (Brogden, 2005)

Following are examples of AMPs classified using their mechanism of action and intracellular targets –

Model of antimicrobial activity	Synonym	Examples of peptides
Transmembrane pore-forming mechanisms		
Toroidal pore	Wormhole, disk	Magainin 2[70], protegrin-1[62], melittin[55,81], LL-37[65] and MSI-78[90]
Carpet		Dermaseptin S[85], cecropin[156,157], melittin[158], caerin 1.1[159] and ovispirin[64]
Barrel stave	Helical-bundle model	Alamethicin[61,81]
Modes of intracellular killing		
Flocculation of intracellular contents		Anionic peptides[30]
Alters cytoplasmic membrane septum formation		PR-39[109], PR-26[109], indolicidin[110] and microcin 25[111]
Inhibits cell-wall synthesis		Mersacidin[112]
Binds nucleic acids		Buforin II[113] and tachyplesin[114]
Inhibits nucleic-acid synthesis		Pleurocidin[115], dermaseptin[115], PR-39[73], HNP-1, -2[44] and indolicidin[110]
Inhibits protein synthesis		Pleurocidin[115], dermaseptin [115], PR-39[73], HNP-1, -2[44] and indolicidin[110]
Inhibits enzymatic activity		Histatins[117], pyrrhocoricin, drosocin and apidaecin[118]

Table 3 Examples of AMPs with their mechanism of action and intracellular effects (Brogden, 2005)

7. Production of AMPs –

Though the AMPs are promising candidates for treating AMR, their application is limited by the production yields. Currently the AMPs are being synthesized using either biological or chemical synthesis.

Chemical Synthesis: The broad categories of the chemical synthesis are – Solution phase synthesis, Solid phase synthesis and hybrid approaches. Solution phase synthesis is commonly used for small to medium peptides whereas; solid phase synthesis is employed for larger peptides. The hybrid synthesis combines the properties of solid and solution phase synthesis (da Cunha et al., 2017). Though the chemical synthesis makes it easy to have tailor-made proteins, using this approach for a large-scale production is not preferred. Some of the drawbacks of the system include – Limitation on the length of peptides to be synthesized, hydrophobic proteins not synthesized effectively and high cost of production.

Biological synthesis: To overcome the limitations of chemical synthesis, biological synthesis is used. This includes expression of heterologous proteins in genetically engineered plant/animal cell lines, plants and micro-organisms. The key benefits of using biological systems for synthesis are – large scale production, no limit on the length of the peptides to be produced and availability of post translational modifications. However, this method also has drawbacks such as – tedious engineering processes, toxicity to the producer, proteolysis by the host and costly downstream processing.

The industrial applications of the AMPs will be best seen only if the production is increased on a large scale. Hence, efforts are being made to improve the yield using biological synthesis. For this the most explored microbial host includes – *E. coli* and industrially relevant yeasts like – *Yarrowia, Pichia* and *S. cerevisiae*. The yeast systems have proven to be better than *E. coli*. for reasons in mentioned in the table below.

Comparison of Different Biological Systems for Heterologous Protein production

	Higher Eukaryotes		Yeast		Bacteria
Ease of modification	Moderate		Simple		Simple
Cultivation	Slow growth rate and expensive complex media required		Fast robust growth, defined complex media		Fastest growth, defined minimal media
Contamination	Risk of viral contamination, viral clearance required		Little risk of endotoxins or viral DNA		Endotoxin presence requires through purification and possible phage infections
Post-translational modifications (PTM)	Closely resembles human PTM, usually mixtures of several glycoform variants		Most human PTMs achievable but some differences in the glycosylation pattern, hyper annosylation engineered strains can achieve human glycoforms and high uniformity		Limited set of human PTMs, difficult to achieve
Protein yields and secretory properties	High yields, high secretion capacities, high specific productivity		High yields, secretory capacities depending on the secretory signals linked to the protein of interest		High expression capacities, secretion mostly inefficient, extensive purification and downstream processing required
Most commonly used species	Mammalian cells	Insect cells	*P. pastoris*	*S. cerevisiae*	*Escherichia coli*
Recently approved biopharmaceuticals[a]	32	2	2	4	17
Additional information and specific differences between the host species of the same class	Commonly used cell lines CHO (chinese hamster ovary) BHK (Baby hamster kidney) Murine myeloma derived NSO, SP2O and HEK293	*Baculo* virus-based system most commonly used for transfection	Efficient and selective secretion, often higher protein titers than *S. cerevisiae* Crabtree negative leading to high cell densities **GRAS status –** Hypermanosylation is less pronounced in *P. pastoris* and critical terminal alpha 1,3 mannose linkages were not observed, engineered strains providing fully humanized glycosylation not available in *S. cerevisiae*	Important eukaryotic model, high molecular and cell biological knowledge, Crabtree positive leading to ethanol production	Fastest efficient expression system Inexpensive Well established processes for mass production Formation of inclusion bodies leads to misfolding of proteins and requires expensive refolding Inefficient acetate metabolism may hamper high cell density cultivation of some strains

Table 4 Comparison of Different Biological Systems for Heterologous Protein production

Modified from (Vogl et al., 2013)

8. **Applications of AMPs –**The AMPs with diverse properties and varieties have multiple applications in different industries.

   Pharmaceutical industry – The pharmaceutical industry is constantly in search of newer alternatives to the traditionally used antibiotics. In addition to antimicrobial activity these peptides participate in many cellular functions including chemotaxis, wound healing, and cancer therapies (Li et al., 2012). They can be also used to coat the medical devices so as to prevent the biofilm formation. As AMPs can discriminate between bacteria, they can be used in pathogen detection kits. AMPs show promising characteristics so as to employ them in vaccinations. Chemical fungicides show harmful effects on environment and human health due to their long- lasting effects and accumulation in the human body, AMPs of low toxicity become a good candidate for plant disease control. For example, application of Ace-AMP1 on tomato leaves showed that the recombinant protein conferred strong resistance to the tomato pathogen *A. solani* and could be used as an effective fungicide (Li et al., 2012).

   Food Industry – Because of the potential toxicity of the chemical preservatives, food industry is in search of the preservatives from natural sources. AMPs can also be quality booster for food as they can activate mechanisms of cellular and adaptive immunity. Some AMPs, such as LL-37, can function as potent immune regulator acting as chemokines and/or inducing cytokine production (Li et al., 2012). Antimicrobials from microbial sources, especially nisin, find application in a number of foods such as milk, orange juice, and tomato juice, and for increasing the shelf life of chicken meat without altering sensory properties of the product. Film-coating applications have been reported for meat, fish, poultry, bread, cheese, fruits, and vegetables (Tiwari et al., 2009).

   Other – Alternatively AMPs can be used in waste water treatment, treating infections in aqua culture, hydroponics, infections in birds and other pet animals.

Rationale

Antimicrobial Resistance (AMR) has become a problem of great concern due to the indiscriminate use of antibiotics and adaption of microorganisms to the drugs employed. AMR is the biggest threat to the global health, food security and development today. Hence there is considerable demand of the newer antimicrobial agents.

Antimicrobial Proteins or peptides (AMPs) are promising new candidates for use in multiple industries. They can be modified easily; have shown encouraging results against drug resistant organisms and act on the bacterial membranes, thus, being propitious candidates. They can be naturally found or synthesized. Exploring the antimicrobial proteins or peptides from different plant, animal or microbial sources may give us potential agents to use commercially against pathogens. This study will be focusing on finding out the different natural antimicrobial peptides from unexplored sources like rumen gut microbiota to deal with the microorganisms of concern for different industries like food, therapeutics, aquaculture etc. These AMPs will be produced using industrial work horses like *E. coli, Yarrowia, Pichia, S. cerevisiae* or other related organisms. The biological synthesis of the AMPs will result in improving yields for wider applications.

Aim

Screening, production and characterization of novel antimicrobial protein/s

Objectives

1. To screen different AMPs of interest to identify a template
2. To Clone the genes of interest from natural or synthetic DNA in an appropriate and industrially important host
3. To check the expression of the desired AMP/s
4. To optimize the production of the product on lab scale basis

Materials and Methods

1. **Screening of different AMPs of interest:** Natural antimicrobial peptides from unexplored sources like rumen gut microbiota or other relevant sources will be screened to select natural of interest. Further, their suitability of cloning will be checked using bioinformatics tools. (Mishra et al., 2017)
2. **Cloning of the genes of interest in the vector**

   (Do et al., 2009; Nakamura et al., 2018; Ramani et al., 2015; Soden et al., 2002; Yu et al., 2009)

   a. **Primer designing & Vector selection**

   Considering the product of interest, primers for PCR amplification will be designed using tools provided by NCBI, Genecript etc. One of the commercially available vectors for cloning will be selected and ordered.

   b. **PCR amplification of genes of interest:** The genes of interest will be amplified using PCR. The amplified product will be sequence verified.

   c. **Restriction digestion and ligation:** The sequence verified PCR amplified product and vector selected will be digested using selected restriction enzymes. Digested vector and PCR amplified gene/genes of interest will be ligated.

   d. **Transformation of host/s and screening of transformants:** The ligated vector will be transformed into *E. coli* or suitable yeast host using appropriate method. The cells bearing genes of interest will be screened using appropriate selectable markers. The transformants obtained afterwards will be confirmed for the sequence integrated.
3. **Check the expression of desired AMP/s:** Different methods will be used to check presence and activity of the cloned gene/genes Out of the different clones obtained, clone expressing more amount of the required AMP will be further selected for the lab scale optimization.
4. **Lab scale production optimization:** Different parameters such as; temperature, aeration, time and concentration of inducers will be worked out by design of experiments for optimum production of the AMP (Córdoba-Ruiz et al., 2009; Lee et al., 2013; Zhao et al., 2008).

Statistical Analysis Plan

For the cloned AMP, data will be obtained based on various parameters. In order to check the effectivity of the AMP, it's inhibition will be compared to that of the standard antibiotics used against standard organisms. Optimization of Lab-scale production will be done using parameters like: inducer concentration, time of incubation, Temperature of incubation etc. All these parameters will be monitored in order to get maximum AMP concentration per litre of the media used. The sample size of around four to six replicates will be considered for this.

Quantitative data collected for the variables like inhibition zones formed by the cloned AMP, maximum protein concentration produced (using factors like inducer concentration, time and temperature of incubation etc) will be summarized using **Descriptive statistics like Mean, Standard Deviation, Coefficient of variation. graphical methods. Parametric tests of significance will be used** wherever applicable**.**

The **Qualitative data** obtained (E.g., presence or absence of protein production or inhibition) can be analysed using **count, percentage, graphical methods like pie charts etc and non-parametric tests of significance** wherever applicable.

Expected Outcomes

The AMPs of industrial importance will be identified with the thorough mining of the literature. Accurately designed strategies, will result in creating clones of commercially useful workhorse/s expressing AMP/s of interest. Detection of product activity will help in determining the efficiency of the cloning. Further, this will also help to choose most suitable and higher product yielding clone for lab scale optimization. Lab scale set up for the production of the cloned product will help in determining its suitability for industrial applications.

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